Available online at www.sciencedirect.com
ScienceDirect
Plant Physiology and Biochemistry 45 (2007) 287–292
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research article

Recovering from iron deficiency chlorosis in near-isogenic soybeans: A microarray study

Jamie A. O'Rourke [a], Michelle A. Graham [b], Lila Vodkin [c], Delkin Orlando Gonzalez [c], Silvia R. Cianzio [d], Randy C. Shoemaker [b,*]

[a] Department of Genetics, Developmental and Cellular Biology, Iowa State University, Ames, IA 50011, USA
[b] USDA-ARS, Corn Insect and Crop Genetics Research Unit, Agronomy Department, Iowa State University, G-401 Agronomy Hall, Ames, IA 50011, USA
[c] Department of Crop Sciences, University of Illinois Urbana-Champaign, Urbana, IL 61801, USA
[d] Agronomy Department, Iowa State University, Ames, IA 50011, USA

Available online 14 March 2007

Abstract

Iron deficiency chlorosis (IDC) in soybeans has proven to be a perennial problem in the calcareous soils of the U.S. upper Midwest. A historically difficult trait to study in fields, the use of hydroponics in a controlled greenhouse environment has provided a mechanism to study genetic variation while limiting environmental complications. IDC susceptible plants growing in calcareous soils and in iron-controlled hydroponic experiments often exhibit a characteristic chlorotic phenotype early in the growing season but are able to re-green later in the season. To examine the changes in gene expression of these plants, near-isogenic lines, iron efficient PI548553 (Clark) and iron inefficient PI547430 (IsoClark), developed for their response to iron deficiency stress [USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network − GRIN. (Online Database) National Germplasm Resources Laboratory, Beltsville, MD, 2004. Available: http://www.ars.grin.gov/cgi-bin/npgs/html/acc_search.pl?accid=PI+547430. [22] were grown in iron-deficient hydroponic conditions for one week, then transferred to iron sufficient conditions for another week. This induced a phenotypic response mimicking the growth of the plants in the field; initial chlorosis followed by re-greening. RNA was isolated from root tissue and transcript profiles were examined between the two near-isogenic lines using publicly available cDNA microarrays. By alleviating the iron deficiency stress our expectation was that plants would return to baseline expression levels. However, the microarray comparison identified four cDNAs that were under-expressed by a two-fold or greater difference in the iron inefficient plant compared to the iron efficient plant. This differential expression was re-examined and confirmed by real time PCR experimentation. Control experiments showed that these genes are not differentially expressed in plants grown continually under iron rich hydroponic conditions. The expression differences suggest potential residual effects of iron deficiency on plant health.

Keywords: Iron deficiency chlorosis (IDC); Microarray; Hydroponics; Soybean; Real time PCR (RT-PCR)

1. Introduction

Iron deficiency chlorosis (IDC) results in substantial yield loss throughout the upper Midwestern farmlands each year. The high pH of calcareous soils hinders the formation of the Fe^{+2} ion required for plant uptake. With little to no biologically available iron, plants develop a characteristic interveinal chlorotic patterning of the leaf tissue. If iron-deficient growth conditions are not alleviated, the plant will suffer from stunted growth and reduced yield [9]. Often, the chlorotic phenotype appears early in the growing season and disappears as the plants continue to grow and mature throughout the growing season [11]. Long lasting effects of periods of iron deficiency stress can be observed in the decreased yield of plants that suffered from iron limiting conditions early in the growing season. This carryover effect of brief periods of iron deficiency stress on yield suggests that the genetic changes occurring

* Corresponding author. Tel.: +1 515 294 6233; fax: +1 515 294 9359.
E-mail address: rcsshoe@iastate.edu (R.C. Shoemaker).

0981-9428/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2007.03.008

early in the plant development have a continuing effect later in the plants' life cycle.

Quantitative trait phenotypes are a result of the combined effect of environmental and genetic factors. IDC is an extremely quantitative trait with a large environmental component that makes traditional field studies problematic [5, 7, 14]. However, previous IDC studies have identified a hydroponics system that allows plants to be grown in a manner, whereby the environment is controlled. In replicated trials, the same iron deficiency QTLs are identified in soybean plants grown hydroponically and in field studies [14]. Therefore, this hydroponics system has proven to be a reliable method to examine gene expression of soybean plants suffering from iron deficiency chlorosis [14].

In this study we examined the gene expression profiles of iron efficient and iron inefficient near-isogenic lines (NILs) grown in the hydroponics system. After one week in hydroponic iron-deficient conditions, at which point differential chlorosis was observed between the iron efficient and inefficient NILs, plants were transferred to iron sufficient conditions for one week, after which both iron efficient and inefficient plants appeared green and healthy. Manipulation of the iron content of the hydroponics system allowed us to mirror the phenotypic response of plants in the field. Transcripts with altered expression levels between iron efficient and iron inefficient plants may be indicative of the long residual effects of iron deficiency on soybean plant health.

2. Results

Transcript levels of near-isogenic soybeans, iron efficient Clark (PI548533) and iron inefficient IsoClark (PI547430) were compared by microarray analysis. Plants were grown in hydroponics under iron limiting conditions (50 μM Fe $(NO_3)_3$) for one week, at which time the inefficient line expressed a chlorotic phenotype (Fig. 1). Plants were then transferred to iron sufficient conditions (100 μM $Fe(NO_3)_3$) for one week, at which time both genotypes were equally green. RNA was then extracted from root tissue of both iron efficient and iron inefficient plants. The RNA was fluorescently labeled and hybridized to cDNA microarray slides containing 9728 cDNAs [25] in a balanced dye swap design. A comparison of four biological replicates, with two technical replicates each, for a total of eight microarray hybridizations, revealed four genes exhibiting directionally consistent differential expression on six arrays. These four genes exceeded a two-fold difference of expression between the two genotypes on six of the eight slides (Table 1). All four of the transcripts showed reduced expression levels in the iron inefficient plants in comparison to the iron efficient plants.

As a control, NILs grown in iron sufficient hydroponic solutions (100 μM $Fe(NO_3)_3$) for two weeks were also harvested and analyzed on cDNA slides containing the 9728 genes above, plus an additional 9272 genes. A comparison of three biological replicates with two technical replicates apiece, for a total of six total hybridizations, was analyzed. Using the same stringency

Fig. 1. Differential chlorosis of iron efficient (Clark) and iron inefficient (Iso-Clark) grown in 50 μM $Fe(NO_3)_3$ hydroponic conditions. Chlorosis patterning of iron efficient and iron inefficient plants grown in hydroponics system under 50 μM $Fe(NO_3)_3$. Note the severe interveinal chlorotic response of the iron inefficient plant (Isoclark) compared to the iron efficient plant (Clark). The differential chlorosis response suggests that while the plants have been subjected to the same treatments, they have different tolerances or responses to low iron conditions.

levels as the iron recovery experiment, directionally consistent differential expression on six arrays, we observed no differential expression between the two near-isogenic lines grown continually under iron sufficient conditions for any of the 9728 genes examined in the iron recovery experiment or any of the 9272 additional genes analyzed. However, if stringency levels are lowered to five of six arrays, a small number of genes differentially expressed between the two lines are observed. The four genes identified in this experiment were not among that group (see supplemental data at http://soybase.org/publication_data/ORourke/IronStressRecovery/index.html). The lack of differential expression between the iron efficient and inefficient plants grown under continual iron sufficient conditions has also been observed in an Affymetrix gene chip experiment (O'Rourke and Shoemaker, unpublished data). Thus, the differential expression seen under the iron recovery conditions is likely a result of the NILs' differential response to the changing iron environment.

The expression patterns identified in the microarray experiment were confirmed by reverse transcriptase real time PCR (RT-PCR) (Table 1). As observed with the microarray data, iron inefficient plants had lower expression levels than iron efficient plants (see example, Fig. 2, Table 1). Dissociation curve analysis confirmed that the RT-PCR reaction amplified a single product for each NIL/gene combination. However, a shift in melting temperature between the iron efficient and iron inefficient plants for the asparagine aminohydrolase gene encoded by Gm-c1028-5479 (Fig. 3) suggests sequence differences in the coding region for the gene.

The GenBank accessions of the four EST sequences differentially expressed under the iron recovery conditions were queried against The Institute for Genomic Research (TIGR)

Table 1
Expression levels and annotations for each of the four differentially expressed ESTs identified from plants grown under iron recovery hydroponic growth conditions

EST designation	Federated ratio	TIGR TC	UniProt annotation	*E*-value	RT-PCR cycle differences	Fold-change in gene expression
Gm-c1028-8295	0.284	Singleton	Hypothetical	2e−25	1.30	3
Gm-c1028-7992	0.299	TC221258	Ribophorin	6e−85	1.84	4
Gm-c1028-6182	0.290	TC209369	Protein Kinase	2e−44	1.02	2
Gm-c1028-5479	0.298	TC217977	Asparagine aminohydrolase	1e−61	8.53	256

The level of differential expression is designated by the federated ratio, which is calculated by adding all inefficient expression levels together and dividing by the sum of efficient expression levels. Ratios below a 0.5 indicate a two-fold or greater expression level difference between the iron inefficient genotype and iron efficient genotype. In all cases expression levels in the inefficient genotype were less than expression levels in the efficient genotype. To provide a functional annotation of the EST sequence, the TIGR TC containing the *Glycine max* EST was identified and a tBLASTx was performed on the TIGR TC against the UniProt database. The *E*-value represents the similarity between the UniProt annotation and the TIGR TC sequence. The RT-PCR cycle difference is the difference of the NILs amplification of the target transcript in crossing the amplification threshold after normalization with tubulin. The fold-change in gene expression is the calculated change of the expression difference of the target transcript between the two NILs as represented by the difference in amplification.

soybean gene index (Version 12.0 [17]) to identify the tentative consensus (TC) sequence containing the EST of interest. BLASTX [1] was used to compare the TCs to the Uniref100 protein database (February 2006 [2]) and assign putative function (Table 1). In addition, the TCs were queried against an in-house database of stress-induced genes collected from the literature. Of the four TCs, only one showed any homology to other stress-induced genes. This dissimilarity reinforces the idea that these genes are involved in the recovery process of iron deficiency. The gene showing similarity to other stress-induced genes, Gm-c1028-6182, was part of a TIGR TC with a UNIREF annotation of a transmembrane protein kinase receptor.

3. Discussion

Iron deficiency stress is known to reduce yield of soybeans even though no visual indication of iron chlorosis can be observed. In this study we identified four genes differentially expressed between the two near-isogenic lines after a return to iron sufficient conditions and a return to normal green phenotypes. These four genes were not differentially expressed under iron-deficient conditions (O'Rourke et al., unpublished data) nor do they represent constitutive differences between the NILs grown under iron sufficient growth conditions.

The three genes with putative functional annotations can be ascribed hypothetical roles in iron deficiency-related

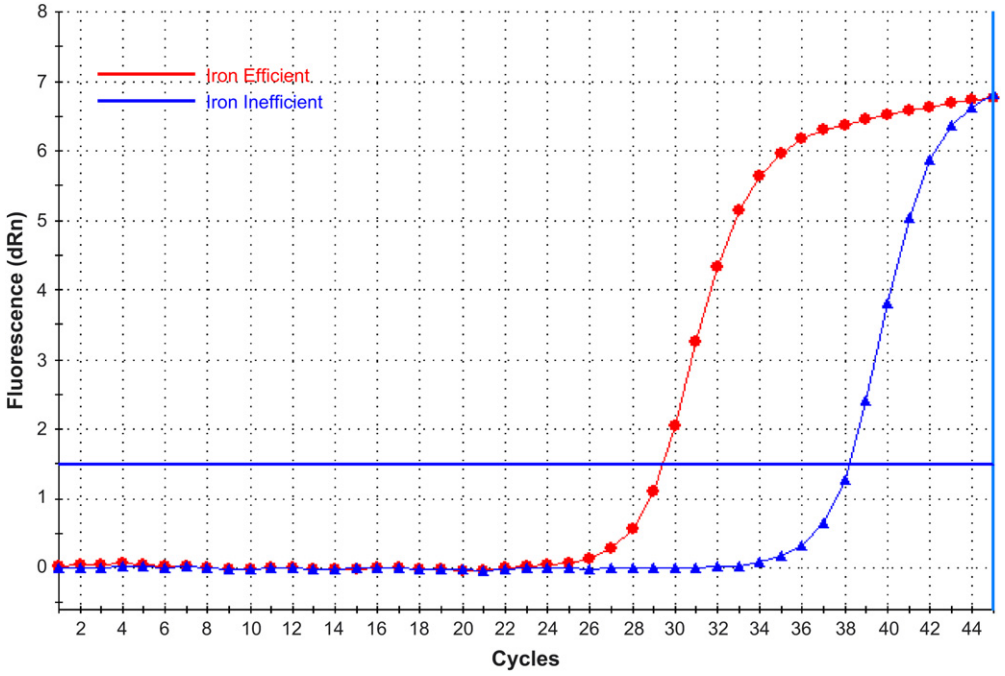

Fig. 2. Amplification plot of Gm-c1-28-5479. Fluorescence levels of SYBR Green covalently bound to the amplicon represented by Gm-c1028-5479. Confirming the microarray results, the expression level of the gene is lower in the Fe-inefficient line than in the Fe-efficient plant. In this case, the difference in Ct values is 8.53 cycles, which translates to a 256-fold difference. This is a greater change in expression levels between Fe-efficient and Fe-inefficient lines than seen in the microarray. It is possible that the microarray was examining expression levels of multiple members of the same gene family, while the RT-PCR is only examining the expression level of one family member.

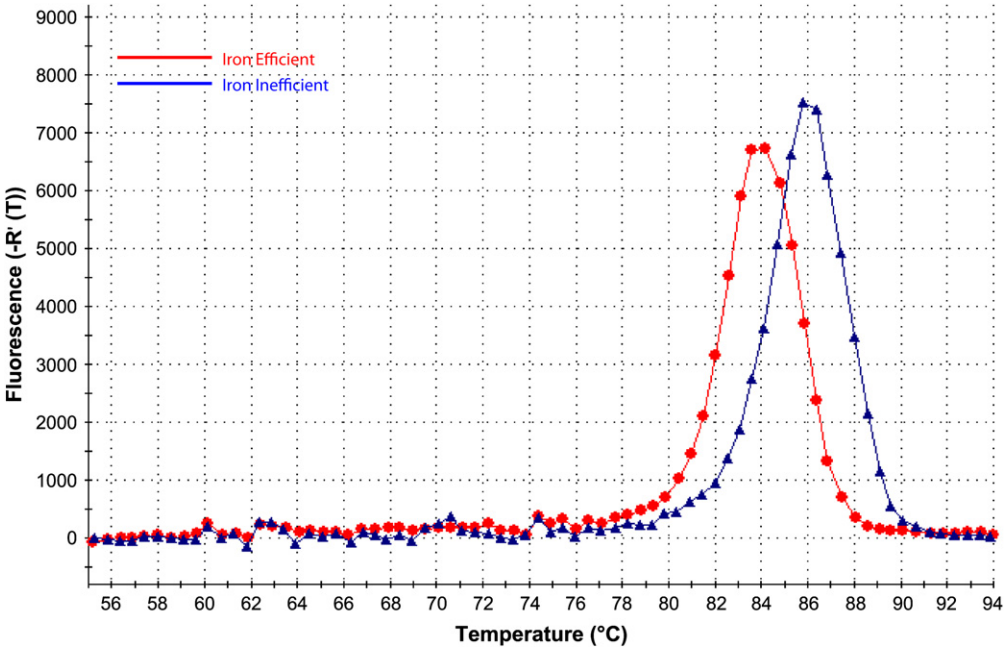

Fig. 3. Dissociation curve of Gm-c1028-5479. Fluorescence levels associated with the melting temperature of the amplicon of Gm-c1028-5479. The shift in melting temperatures between iron efficient and iron inefficient lines suggests that the gene product of Gm-c1028-5479 is genotype dependent. The single peak generated by the degradation of each amplicon confirm only one gene product was amplified for each genotype, reaffirming the sensitivity of the RT-PCR. The different product sizes would suggest that this gene is an excellent candidate for further study in iron efficiency work.

processes. GmTC221258 had a UNIREF annotation of a ribophorin protein (Table 1). Ribophorin proteins are a subunit of the greater oligosaccharyltransferase (OST), which is involved in the glycosylation of proteins entering the endoplasmic reticulum [13, 16]. The ribophorin proteins I and II are thought to be essential components of the OST [13, 16] serving to cross-link the 60 s ribosomal subunit to the endoplasmic reticulum to form the rough ER [18]. The under-expression of this gene in iron inefficient plants compared to iron efficient plants may indicate the production of membranes and vesicles in iron inefficient plants have not yet, and may never, return to the levels seen in iron efficient plants subjected to the same environmental conditions.

The transmembrane protein kinase identified by GmTC209369 may be serving as a receptor protein, possibly recognizing iron availability or other environmental cues [23]. The kinase would then phosphorylate a secondary signaling molecule to initiate a cascade of responses, including root to shoot signaling [19], depending on the amount of iron present. Kinase phosphorylation often serves as a signal to initiate a signaling cascade within the cell. In rice, the over-expression of a transmembrane protein kinase has been shown to induce aluminum tolerance [19]. This mechanism could easily be extended to involve kinase activity in other heavy metal responses. Genes with sequences highly homologous to GmTC209369 have been shown to be differentially expressed under phosphate stress [10] and other forms of abiotic stress [3, 12].

The UNIREF annotation of GmTC217977 suggests that this gene encodes an asparagine aminohydrolase. Asparaginase activity has been shown to provide ammonia as a nitrogen source for all nitrogen containing compounds within the cell. The enzymatic activity has been shown to be upregulated during protein synthesis [4, 15]. Activity levels of asparaginase are highest in roots and nodules [20] so it is not surprising the expression levels of asparagine aminohydrolase would be differentially expressed in the root systems of iron efficient and inefficient plants. Differential expression of the asparagine aminohydrolase suggests that the protein synthesis and nitrogen metabolism pathways in the iron inefficient plant have not returned to the production levels of the iron efficient plant.

The real time PCR confirmation experiments detected greater differential expression between the NILs than was observed with the microarray. This may be because the microarray analysis, through hybridization, reflects the expression level of multiple members of a gene family while the RT-PCR is specific to the individual family member represented by the EST sequence.

The dissociation analysis of Gm-c1028-5479 revealed sequence dissimilarity between the iron efficient and iron inefficient lines. Both genotypes amplified only one PCR product, as shown by the single peak of the dissociation curve (Fig. 3). However, the shift in melting temperatures between the two genotypes suggests that the sequence of the asparagine aminohydrolase gene is longer in the iron inefficient plant than in the iron efficient plant. Alternatively, the near-isogenic lines may have different GC concentrations in the region of the gene being transcribed, or, much less likely, we may be amplifying different members of the same gene family. That the gene encoded by Gm-c1028-5479 appears to have a genotype-dependent coding region makes this gene an excellent candidate for further examination in its role of iron efficiency.

The four candidate genes identified by microarray analysis in this study are excellent candidate genes to study the long-term effects of iron deficiency on soybean. The induction of these genes after returning to an iron sufficient environment suggests that these genes may be directly involved in utilizing available iron. The sequence differences in the asparagine aminohydrolase gene suggested by the RT-PCR dissociation curve suggests that different members of the same gene family may be differentially transcribed in different genotypes possibly resulting in different activity levels within the plants. The lower expression levels of these genes in iron inefficient plants suggests that they may not reach expression levels achieved by iron efficient plants. This reduced expression pattern may reflect reduced activity levels, which, speculating, could explain the reduced yield often seen from plants experiencing iron deficiency chlorosis early in the growing season. This study has identified a small subset of the genes likely involved in this complicated metabolic process. In the future, experiments using larger arrays such as the Affymetrix gene chip may help to identify additional candidate genes.

4. Methods

4.1. Plant growth conditions

Near-isogenic soybean lines, PI548533 'Clark' and PI547430 'IsoClark' [22] were grown in the Ames, IA, USDA greenhouse under 16 h photoperiods. Plants were germinated in sterile vermiculite with distilled deionized water. After one week they were transferred to a DTPA nutrient buffered hydroponics system [6] containing all minerals necessary for normal growth with iron being the only limiting component. Specifically, each 10 L system contained 2 mM $MgSO_4$*$7H_2O$, 3 mM $Mg(NO_3)_2$*$6H_2O$, 2.5 mM KNO_3, 1 mM $CaCl_2$*$2H_2O$, 4.0 mM Ca $(NO_3)_2$*$4H_2O$, 0.020 mM KH_2PO_4, 542.5 μM KOH, 217 μM DTPA, 1.52 μM $MnCl_2$*$4H_2O$, 4.6 μM $ZnSO_4$*$7H_2O$, 2 μM $CuSO_4$*$5H_2O$, 0.20 μM $NaMoO_4$*$2H_20$, 1 μM $CoSO_4$*$7H_2O$, 1 μM $NiSO_4$*$6H_2O$, 10 μM H_3BO_3, and 20 mM HCO_3. A pH of 7.8 was maintained by the aeration of a 3% CO_2:air mixture to each 10 L system. To induce iron deficiency, plants were limited with 50 μM $Fe(NO_3)_3$ for one week after which the iron level was increased to a non-limiting 100 μM $Fe(NO_3)_3$. NILs also were grown constitutively at 100 μM $Fe(NO_3)_3$, as controls. A supplemental nutrient solution containing 16 mM potassium phosphate, 0.287 mM boric acid and 355 mM ammonium nitrate was added daily to all plants to maintain proper plant nutrition. As further visual controls and to ensure that chlorosis was due to iron deficiency stress, A15, an iron efficient plant and T203, iron inefficient plant, were also included with each experimental replication. At the end of this period plants were at the V3 stage [8].

4.2. RNA extraction and microarray hybridizations

The iron recovery experiment was comprised of four biological replicates, each with two technical replicates for a total of eight hybridizations. Samples were hybridized to a spotted slide containing 9728 cDNAs from Gm-c libraries 1021 and 1083, which are root specific. Gene expression patterns of plants for the control experiment were compared across three biological replicates, with two technical replicates apiece, for a total of six hybridizations. Samples for the control plants were hybridized to spotted slides containing the 9728 cDNAs present on the experimental slides plus an additional 9272 cDNAs. The additional genes allowed for a more global analysis of known root transcripts to determine if there are any inherent genetic differences between the near-isogenic lines.

Total RNA was extracted from root tissue of iron recovery plants using a modified phenol chloroform extraction with a lithium chloride precipitation protocol as set forth by the NSF Soybean Microarray Workshop in May of 2000 [24]. Samples were composed of root tissue from four individual plants, all grown in the same hydroponic unit. Samples were further purified using RNeasy kits from Qiagen. RNA was extracted from iron sufficient control plants following the RNA extraction protocol of the Qiagen RNeasy kits. For both methods, each sample yielded 180 μg of purified RNA, 90 μg of purified RNA for each cDNA array of the dye swapped slides.

Purified RNA samples were split into 90 μg aliquots and heated with oligo dT for 10 min. A total of 20 μL of 1× buffer, 10 mM DTT, 500 μM low T dNTPs, 100 μM Cy3 or Cy5 (Amersham Biosciences), and 13 units/μL SuperScriptII (Invitrogen) were added to each sample then placed at 42 °C for 2 h. Remaining RNA was degraded with an RnaseA/H treatment. Labeled Clark and IsoClark cDNA samples were mixed in a balanced dye swap design labeled with PolyA DNA and hybridized for 18 h at 42 °C. After overnight hybridizations, slides were washed (wash 1: 1× SSC, 0.2% SDS, wash 2: 0.2× SSC, 0.2% SDS, wash 3: 0.1× SDS) to remove unbound cDNAs. Slides were scanned with ScanArray Express (Stratagene) and resulting images were overlaid and spots identified by the ImaGene program. An analysis program developed at the University of Illinois [21] was used to identify differentially expressed cDNAs. For our purposes, differential expression is defined as a minimum of a two-fold over or under-expression of the cDNA in IsoClark relative to Clark.

4.3. Real time PCR

For the RT-real time PCR experiments 200 ng of RNA extracted from root tissue served as initial template for each sample. Primers were designed based on available EST sequences from GenBank to produce 250 bp amplicons. Primer sequences were as follows: Gm-c1028-8295: F: GGCCACCATGTAACTTATTC, R: ACTGGCATTGCTGATTGACA; Gm-c1028-7992: F: CACTGTTAAATTGCCTGATGC, R: CTCGCACCAACTCTTTAGC; Gm-c1028-6182: F: CAGTGGGAAAGAATCTTGTCAC, R: GCCATATTCACTGAGAGGTTAC; Gm-c1028-5479: F: GACATTCCAAGGTTGCGTAGGC, R: CGCCATTTCTGTTTCGCTTATGG; tubulin: F: CAATTGGAGCGCATCAAT, R: ATACACTCATCAGCATTCTC. Stratagene's brilliant qRT-PCR kit was used with each 25 μL reaction assembled as described by the Stratagene instruction manual

(Catalog #600532) with 2.5 μL of 50 mM $MgCl_2$, and 2 μL of 50 nM F and R primers. Cycling protocols consisted of a 45 min at 42 °C, 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min at 62 °C, and 30 s at 72 °C. The PCRs were run in the Stratagene Mx3000P followed by a dissociation curve, taking a fluorescent reading at every degree between 55 °C and 95 °C to ensure only one PCR product was amplifying. The Stratagene analysis system established a threshold fluorescence level where amplicon fluorescence levels were statistically higher than background fluorescence; this threshold level is referred to as the Ct value, the cycle at which the samples fluorescence is above threshold. As controls, a passive reference dye was added to each sample, to ensure recorded fluorescence levels were due to SYBR green incorporation. Additionally, each sample was also run in triplicate and each sample was also normalized against tubulin amplification, see primers above, to ensure the differential expression was not due to differing amounts of initial RNA template added to each sample. To be considered differentially expressed, the iron efficient and iron inefficient plants at the same time point had to differ in where they crossed the fluorescence threshold by more than one cycle.

Dissociation analyses of RT-PCR products were conducted by measuring fluorescence levels between 55 °C and 95 °C on a Stratagene MX3000P.

References

[1] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Research 25 (1997) 3389–3402.

[2] R. Apweiler, A. Bairoch, C.H. Wu, W.C. Barker, B. Boeckmann, S. Ferro, E. Gasteiger, H. Huang, R. Lopez, M. Magrane, M.J. Martin, D.A. Natale, C. O'Donovan, N. Redaschi, L.S. Yeh, UniProt: the universal protein knowledgebase, Nucleic Acids Research (2004) D115–D119.

[3] H. Bae, M.S. Kim, R.C. Sicher, H.J. Bae, B.A. Bailey, Necrosis and ethylene inducing peptide from *Fusarium oxysporum* induces a complex cascade of transcripts associated with signal transduction and cell death in *Arabidopsis*, Plant Physiology 141 (2006) 1056–1067.

[4] A. Casado, J.L. Caballero, A.R. Franco, J. Cardenas, M.R. Grant, J. Munoz-Blanco, Molecular cloning of the gene encoding the L-asparaginase gene of the *Arabidopsis thaliana*, Plant Physiology 108 (1995) 1321–1322.

[5] S.R. Cianzio, W.R. Fehr, I.C. Anderson, Genotypic evaluation for iron deficiency chlorosis in soybeans by visual scores and chlorophyll concentration, Crop Science 19 (1979) 644–646.

[6] B.A. Coulombe, R.L. Chaney, W.J. Wiebold, Bicarbonate directly induces iron chlorosis in susceptible soybean cultivars, Soil Science Society American Journal 48 (1984) 1297–1301.

[7] B.W. Diers, S.R. Cianzio, R.C. Shoemaker, Possible identification of quantitative trait loci affecting iron efficiency in soybean, Journal of Plant Nutrition 15 (1992) 2127–2136.

[8] W.R. Fehr, C.E. Caviness, Stages of soybean development, Special Report 80, Cooperative Extension Service, Iowa State University, CODEN:IWSRBCC(80), 1977, pp. 1–12.

[9] D.M. Froelich, W.R. Fehr, Agronomic performance of soybeans with differing levels of iron deficiency chlorosis on calcareous soil, Crop Science 21 (1981) 438–441.

[10] M.A. Graham, M. Ramirez, O. Valdes-Lopez, M. Lara, M. Tesfaye, C.P. Vance, G. Hernandez, Identification of candidate phosphorus stress induced genes in *Phaseolus vulgaris* L. through clustering analysis across several plant species, Functional Plant Biology 33 (2006) 789–797.

[11] N.C. Hansen, M.A. Schmitt, J.E. Anderson, J.S. Strock, Soybean: iron deficiency of soybean in the upper midwest and associated soil properties, Agronomy Journal 95 (2003) 1595–1601.

[12] X.J. He, R.L. Mu, W.H. Cao, Z.G. Zhang, J.S. Zhang, S.Y. Chen, AtNAC2 a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development, The Plant Journal 44 (2005) 903–916.

[13] A. Helenius, M. Aebi, Roles of N-linked glycans in the endoplasmic reticulum, Annual Review of Biochemistry 73 (2004) 1019–1049.

[14] S. Lin, J.S. Baumer, D. Ivers, S. Cianzio, R. Shoemaker, Field and nutrient solution tests measure similar mechanisms controlling iron deficiency chlorosis in soybean, Crop Science 38 (1998) 254–259.

[15] K. Michalska, G. Bujacz, M. Jaskolski, Crystal structure of plant asparaginase, Journal of Molecular Biology 360 (2006) 105–116.

[16] A.V. Nikonov, G. Krelbich, Organization of translocon complexes in ER membranes, Biochemical Society Transactions 31 (2003) 1253–1256.

[17] J. Quackenbush, J. Cho, D. Lee, F. Liang, I. Holt, S. Karamycheva, B. Parvizi, G. Pertea, R. Sultana, J. White, The TIGR Gene Indices: analysis of gene transcript sequences in highly sampled eukaryotic species, Nucleic Acids Research 29 (2001) 159–164.

[18] M.G. Rosenfeld, E.E. Marcantonio, J. Hakimi, V.M. Ort, P.H. Atkinson, D. Sabatini, G. Kreibich, Biosynthesis and processing of ribophorins in the endoplasmic reticulum, The Journal of Cell Biology 99 (1984) 1076–1082.

[19] M. Shivaguru, B. Ezaki, Z.H. He, H. Tong, H. Osawa, F. Baluska, D. Volkmann, H. Matsumoto, Aluminum-induced gene expression and protein localization of a cell wall-associated receptor kinase in *Arabidopsis*, Plant Physiology 132 (2003) 2256–2266.

[20] J. Streeter, Asparaginase and asparagine transaminase in soybean leaves and root nodules, Plant Physiology 60 (1977) 235–239.

[21] F. Thibaud-Nissen, R.T. Shealy, A. Khanna, L.O. Vodkin, Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean, Plant Physiology 132 (2003) 118–136.

[22] USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network – GRIN. (Online Database) National Germplasm Resources Laboratory, Beltsville, MD, 2004. Available: http://www.ars.grin.gov/cgi-bin/npgs/html/acc_search.pl?accid=PI+547430.

[23] J.A. Verica, L. Chae, J. Tong, P. Ingmire, Z.H. He, Tissue-specific and developmentally regulated expression of a cluster of tandemly arrayed cell wall-associated kinase-like kinase genes in *Arabidopsis*, Plant Physiology 133 (2003) 1732–1746.

[24] L. Vodkin, R. Shealy, S. Clough, R. Philip, T. Raph, L. Shoop, S. Bergman, R. Staggs, M. Karo, NSF Soybean Microarray Workshop, University of Illinois, 2000.

[25] L. Vodkin, A. Khannaa, R. Shealy, S.J. Clough, D.O. Gonzalez, R. Philip, G. Zabala, F. Thibaud-Nissen, M. Sidarous, M.V. Stromvik, E. Shoop, C. Schmidt, E. Retzel, J. Erpelding, R.C. Shoemaker, A.M. Roxrigues-Huete, J.C. Polacco, V. Coryell, Paul Keim, G. Gong, L. Liu, J. Pardinas, P. Schweitzer, Microarrays for global expression constructed with a low redundancy set of 27,000 sequenced cDNAs representing an array of developmental stages and physiological conditions of the soybean plant, BMC Genomics 5 (2005) 73. doi:10.1186/1471-2164-5-73.